CHARACTERIZATION OF OCA-B INDUCTION IN NAÏVE CD4+ T CELLS

by

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A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Laboratory Medicine and Biomedical Science

Department of Pathology

The University of Utah

August 2015

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The University of Utah Graduate School

STATEMENT OF THESIS APPROVAL

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ABSTRACT

Oct1 and its transcriptional cofactor OCA-B are required for memory $CD4^+$ T cell development. This study describes OCA-B induction patterns in naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells have no endogenous expression of OCA-B. After 6 hours of in vitro stimulation, by plate bound CD3ε antibodies and CD28 antibodies in media, T cells begin expression of OCA-B. OCA-B expression increases gradually over time with continued stimulation. Maintenance of OCA-B expression is influenced by Oct1 activity: in the absence of Oct1, OCA-B levels are reduced at long time points following stimulation. Additionally, a novel function of OCA-B positively influencing surface expression of CD44, a surface receptor required for memory T cell survival, has been discovered.

A floxed, conditional cre-driven knockout allele of OCA-B has been generated in C57/BL6 mice. This allele will prove to be an invaluable tool for researching further in vivo functions of OCA-B.

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ACKNOWLEDGEMENTS

First, I have to give the obvious big thank you to my family and friends for their unconditional love and support in these last few years. I wouldn't be where I am without them, especially without the push provided by my better half, Wency Lam. I love you all and appreciate each of you more than I could ever show.

Second, my gratitude goes towards people who helped me find my way to and through the program. To JoAnn Fenn, Diana Wilkins and everybody in the Laboratory Medicine/Biomedical Sciences offices: thank you for your assistance in finding the end of the tunnel. To Dr. Dylan Miller, Dr. Evelyn Gopez and Dr Brian Avery: thank you for your letters of recommendation and guidance you've provided to me in the years before I joined the program.

Third, my committee members: Dr. Dean Tantin, Dr. Matthew Williams and Dr. Ryan O'Connell. Thank you all for being patient with me over these last few months in finishing my project. Matt: thank you for the knowledge you've provided in helping me understand the immunological aspects. Ryan: thank you for becoming my last-minute committee member without hesitation. Dean: thank you for generously taking me into your lab and giving me the opportunity to work under your tutelage. I will carry what I've learned here with me for the rest of my life.

Lastly, I must thank the members of the Tantin lab: Arvind Shakya, Zuolian Shen,

Jillian Jafek, Karina Vazquez-Arreguin and Pei-yi Tai. You all have been very accommodating these last few years and it has been a privilege to work with each of you. A special thank you goes to Arvind, who provided me with a majority of the hands on training to reach my goals. I hope the best for you all in your future endeavors.

CHAPTER 1

INTRODUCTION

Octamer transcription factor Oct1

A subset of POU (Pit-1, Oct1/2, Unc-86) domain transcription factors, referred to as Oct proteins or octamer transcription factors, recognize the highly conserved 8 base pair sequence [ATGC(A/T)AAT], known as the "octamer motif" (1). The class V Oct protein Oct4 (*Pou5f1*, Oct3), a key factor in induced pluripotent stem cell development, is perhaps the most renowned, but other Oct proteins have also garnered more focus over the years (2).

Oct1 (*Pou2f1*) is a class II octamer transcription factor which contains a POU specific box (POU_S) for recognition of the 5' end of the octamer motif, and a POU-homeodomain (POU_H) for recognition of the 3' end (3–6). A close relative to Oct 4, Oct1 is a ubiquitously expressed transcription factor involved in gene activation, repression and antirepression (or "poising") (3, 7, 8).

Modifications to Oct proteins affect stability, DNA binding capabilities, cofactor association, and nuclear association (3). Oct1 is modified by phosphorylation and ubiquitylation during mitosis (9). Under various overgrowth and metabolically stressed conditions, Oct1 undergoes p-GlcNAcylation modification (9, 10). During exposure to

oxidative and genotoxic stress, Oct1 is dynamically phosphorylated in order to modulate gene expression to promote cell survival (10).

Early findings have shown Oct1 to be involved in housekeeping gene expression, specifically influencing histone H2B transcription (11). Since its discovery, Oct1 has been found to target other functional gene groups, including various developmental regulators (i.e., *Pax6*, *Hoxb1*) and metabolic genes (i.e., *Pcx*, *Hk1*) (7). Oct1 also interacts with promoter regions of inflammatory cytokines *Il2, Il3, Il5, GM-CSF*, and *Il4* (12, 13). A single gene can be modified into an active state, repressed state or poised state via Oct1 mediated histone modifications at gene promoter sites, as seen at the T cell *Il2* (3, 8).

Oct1 has been found to be involved in a multitude of biological functions. $Oct1^{-/-}$ mice are embryonic lethal, with notable anemia attributed to deficiencies in erythropoiesis and β-globin gene expression, suggesting Oct1 plays a key role in red blood cell development (14). In human mesenchymal stem cells prematurely aged via prelamin-A accumulation, impaired Oct1 activity is described to possibly contribute to cellular dysfunction (15). Oct1 has been shown to work as a stress sensor that modulates gene expression in response stressors such as overgrowth conditions, genotoxic stress, gamma radiation or oxidative stress (7, 16, 17). Despite being dispensable for induced pluripotent stem cell development, Oct1 has been shown to be elevated in both somatic and cancer stem cells (2, 18). Specifically, Oct1 controls stem cell phenotype functions, potentiates tumor cell engraftment, and regulates hematopoietic transplant engraftment (18).

Clinically, aberrant expression of Oct1 has been correlated with various forms of cancer. POU homeobox genes, including Oct1 specific targets, are overexpressed in

patient breast cancer samples as well as $CD24^{LO}CD44^{HI}$ breast cancer initiating cells (18, 19). Patient prostate cancer samples found with high levels of Oct1 expression have been correlated with lower patient survival rates (20). Cervical and gastric cancers both show similar phenomena, with the latter linking Oct1 activity to potentiated ERK phosphorylation associated with gastric cancer development (21, 22).

Oct1 transcriptional coactivator OCA-B

Oct proteins, including Oct1, operate under the influence of various mediators to modulate activity (3). OCA-B (*Pou2af1*, Bob.1, OBF-1) is a lymphocyte specific coactivator that functions as a modulator for inflammatory responses in B and T cells via Oct1 and Oct2 (3, 23–27).

OCA-B is a 256 amino acid protein that contains a stretch of seven amino acids near the N-terminus vital for contacting POU domain of Oct1, and the C-terminal domain required for transcriptional activation (23). Modifications to an acidic region of the C-terminal domain regulate OCA-B stability and activity (28). The Oct1/OCA-B complex prefers adenine at the fifth position of the octamer motif (29). While Oct1 is bound to DNA, OCA-B makes contacts with the POU_S and POU_H domains to stabilize Oct1 on the target octamer motif in what is described as a “molecular clamp” (30).

In B cells, OCA-B has been found as two isoforms generated from the same *Pou2af1* gene: p34 and p35 (31). The p35 isoform, derived from a p40 precursor isoform expressed upstream of the p34 locus, is anchored to the outer membrane until signaled for nuclear import (32). The p34 isoform is imported directly into the nuclear membrane posttranslation and functions as a more potential transcriptional activator than its

counterpart (32). Overexpression of the p34 isoform causes defects at early stages of B cell development resulting in reduced peripheral follicular B cells and reduced pre-B cells in bone marrow (33). The follicular B cells produced from OCA-B overexpression show defects in immune responses due to irregular expression of inflammatory genes (e.g., *HLA-DRB1*, *Gadd45β*, *TCF12*) (33).

Due to the high endogenous level in B cells, OCA-B was originally thought to be a B cell specific transcriptional cofactor of Oct1 and Oct2 (24). Initial findings revealed OCA-B strongly associating with immunoglobulin promoters in B cells, implicating the cofactor as a regulatory element involved in immunoglobulin production (24, 26, 27). Loss of OCA-B results in reduced V-D-J recombination in B cell immunoglobulin kappa genes (34).

Failure to form germinal centers (GCs) is the most profound immunological defect in OCA-B$^{-/-}$ mice (25). GC formation requires B cell expression of OCA-B, independent of functional OCA-B expression in T cells (35). OCA-B expression is upregulated in GC B cells (36). Along with Oct2, B cells express *Il6* in an OCA-B dependent manner during antiviral responses, which stimulates T follicular helper cell differentiation (37). In a similar fashion, OCA-B facilitates B to T cell interactions during humoral immune response for plasma cell maturation (38).

With the focus on OCA-B functionality in B cells, the role of OCA-B in T cells has been largely overlooked. CD4^{+} T cells have no detectable amounts of endogenous OCA-B by northern blot, but RNA production is inducible and peaks at 4 hours with ionomycin/TPA treatment (39). OCA-B$^{-/-}$ mice infected with *Leishmania major* exhibit diminished CD4^{+} Th1 function and elevated CD4^{+} Th2 cytokine production resulting in

higher susceptibility to infection (40). This suggests OCA-B as a possible regulator of Th1/Th2 population homeostasis (40).

Leukemia and lymphoma are two major disease states associated with altered expression of OCA-B (36, 41). In GC derived lymphomas, OCA-B and Oct2 were found to be expressed at significantly higher levels (36, 42). Clinically, Oct2 and OCA-B, along with Pax-5, Bcl-5 and MUM1, have been suggested as diagnostic markers for nodular lymphocyte predominant Hodgkin lymphoma (43). The loss of immunoglobulin expression found in Hodgkin and Reed-Sternberg cells, the hallmark of transformed malignant cells in classical Hodgkin lymphoma, has been linked to loss of Oct2 and OCA-B expression (44). While no correlation of OCA-B/Oct2 over-expression is seen in patient leukemia samples, it has been suggested that lowered co-expression of the transcriptional regulators could be used to predict positive prognosis in acute myeloid leukemia (36, 45). Even though a mechanistic contribution has not been ascertained in these diseases, it is clear that misregulation of OCA-B is involved in these hematopoietic disease states. Further research is required to elucidate any influence OCA-B has in disease development.

$CD4^+$ T cell memory development

During an inflammatory response T cells undergo activation, clonal expansion, and ultimately gain effector function. A small subset of activated T cells is specialized and maintained as a long-term memory reservoir (46). The mechanism of $CD4^+$ memory pool generation is not well understood. It is known that unlike $CD8^+$ cells, T cell receptor (TCR) signaling plays pivotal a role in effector T-helper (Th1) $CD4^+$ T cell memory

development (47). More specifically, sustained interactions between $CD4^+$ T cell TCRs and antigens are essential for development of Th1 memory cells (48).

Long-term survival of memory T cells is dependent on surface CD44 expression (49). It has been shown that engagement of surface CD44 on activated and memory Th1 T cells provides protection from Fas-mediated apoptosis, promoting memory T cell development and long-term survival (49).

A key feature of memory T cells is a robust secondary inflammatory response. In naïve $CD4^+$ T cells, Oct1 represses *Il2* via promoter histone deacetylation mediated by NuRD (8). During activation, *Il2* is modified into an active state via histone demethylation mediated by Oct1 and histone demethylase Jmjd1a (8). After activation, Oct1 and Jmjd1a maintain *Il2* in a poised, demethylated state allowing prompt, robust expression of *Il2* during secondary T cell activation (8). Furthermore, Shakya et al. found that generation of poised gene states of inflammatory genes in T cells requires Oct1 along with its coactivator OCA-B (50). In addition to *Il2*, other genes in $CD4^+$ T cells poised in an Oct1/OCA-B dependent manner include: *Il3, Il17a,* and *Ifng* (50). Overall, these findings show that Oct1 and OCA-B are crucial factors in developing and functioning $CD4^+$ memory T cells (50).

Thesis objectives

The novelty of $CD4^+$ Memory T cell establishment being dependent on OCA-B and Oct1 implores further knowledge of OCA-B expression and functionality within $CD4^+$ T cells.

OCA-B is not detectable in naïve $CD4^+$ T cells (50). Between 6 and 12 hours of

CD3ε/CD28 stimulation, OCA-B is expressed at levels detectable via western blot (50). My first objective was to determine precisely how long naïve $CD4^+$ T cells must be stimulated via CD3ε/CD28 to begin OCA-B expression. OCA-B induction was compared to $CD4^+$ T cell activation marker CD69, as well as surface antigens CD44 and CD62L.

As described, Oct1 modulates many genes under various conditions. Based on this, my second objective was to investigate if Oct1 is involved in the maintenance of OCA-B expression in T cells after primary stimulation.

The final objective involved the generation of mice with floxed OCA-B alleles for conditional OCA-B knockout via cre drivers. Current OCA-B research involving mice models have been accomplished using full genotype OCA-B knockout mice and adoptive transfer models. Conditional OCA-B knockout mice will be an invaluable tool for elucidating the role OCA-B has, not only in memory $CD4^+$ T cell development, but also for examining roles in aforementioned hematopoietic disease states.

CHAPTER 2

OCA-B INDUCTION CHARACTERISTICS

OCA-B induction in naïve $CD4^+$ T cells after 6-8 hours of stimulation

Arvind Shakya of the University of Utah's Tantin Lab, has shown naïve $CD4^+$ T cells isolated from wild-type C57/BL6 mice require at least 12 hours of CD3ε/CD28 stimulation to begin transcription of OCA-B ((50), Figure 2.1 A). In this experiment, Dr. Shakya isolated naïve $CD4^+$ T cells from mice spleens using a negative enrichment protocol, performed in vitro stimulation via plate-bound CD3ε antibodies and CD28 antibodies in media, and isolated protein from each sample for western blot at the designated times (8). I repeated the western blot experiment performed in Figure 2.1 A, but adjustments were made to include stimulation times of 8 hours and 10 hours. OCA-B expression is detectable via western blot in primary $CD4^+$ T cells following 8 hours of stimulation (Figure 2.1 B).

A shortcoming of western blot is the somewhat limited quantifiable data produced. Specifically, western blot looks only at global average expression of a cell population rather than giving a measurement of individual cellular expression. Therefore, flow cytometry was employed to provide quantitative measurement of OCA-B

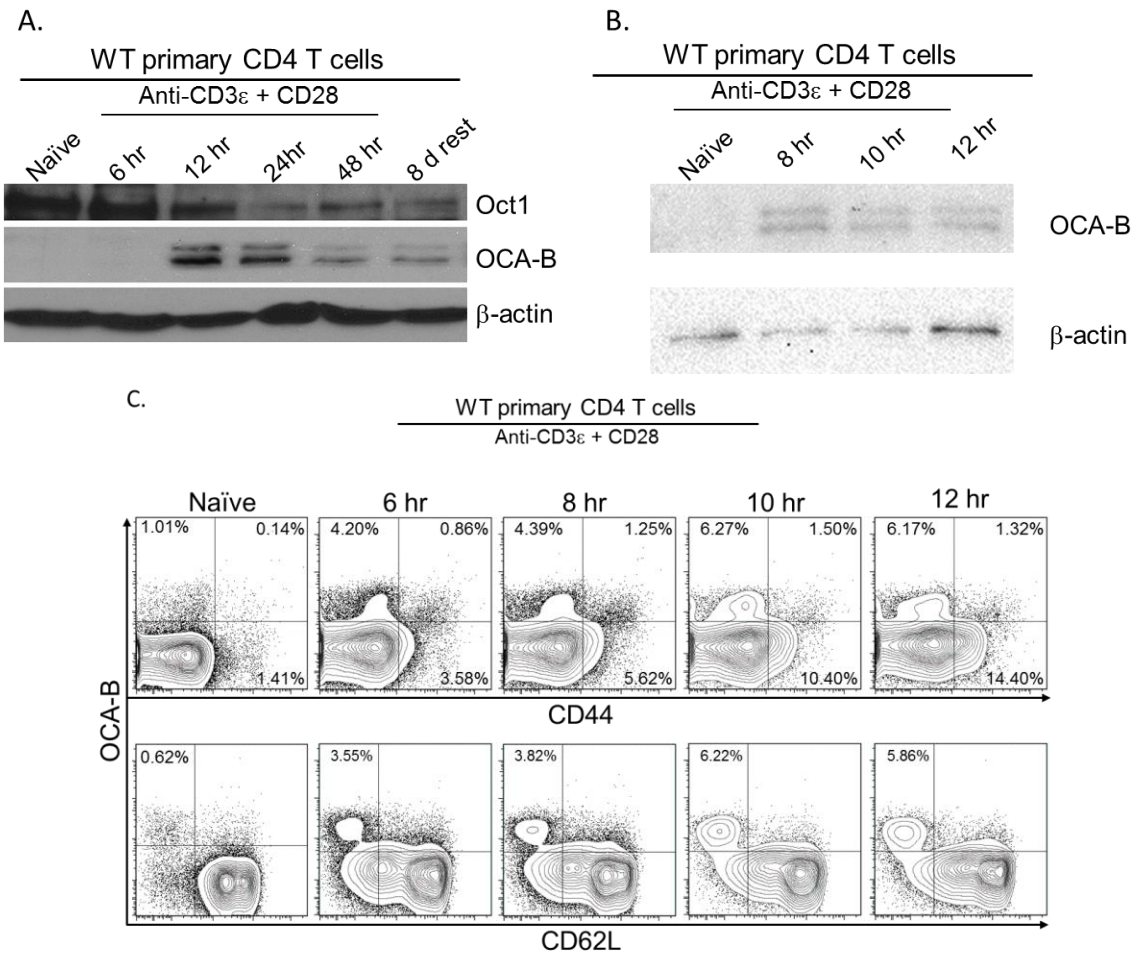

Figure 2.1 OCA-B induction in naïve CD4$^+$ T cells isolated from wild-type C57/BL6 mice stimulated via CD3ε/CD28 antibodies in vitro. A) Western blot analysis of naïve CD4$^+$ T cells stimulated via CD3ε/CD28 at times indicated showing OCA-B induction in naïve CD4$^+$ T cells after 12 hours of stimulation, as performed by Arvind Shakya (50). B) A repeat of the experiment shown in A, using naïve CD4$^+$ T cells isolated from 3 wild-type C57/BL6 mice with shorter stimulation times as indicated. C) Repeat of the experiment shown in A, with naïve CD4$^+$ T cells isolated from 3 wild-type C57/BL6 mice, analyzed via flow cytometry. CD4$^+$, CD8$^-$ gated cells show OCA-B induction after 6 hours of stimulation.

expression. I repeated the experiment seen in Figure 2.1 B, but with a novel intracellular staining protocol for OCA-B. Flow analysis of $CD4^+$, $CD8^-$ gated cells has OCA-B expression seen after only 6 hours of stimulation (Figure 2.1 C). OCA-B expression gradually increases with continued stimulation.

OCA-B induction compared to CD69, CD62L and CD44

CD69 is a surface lectin receptor expressed very early in T cell activation which has been widely accepted as a marker for activated $CD4^+$ T cells (51). Due to its relatively quick induction and implication as an activation marker, it is prudent to compare CD69 expression patterns to OCA-B. I repeated the experimental procedures performed in Figure 2.1, but with shorter stimulation times, as indicated, and CD69 staining included (Figure 2.2 A). OCA-B is found to be expressed slower and less intense compared to CD69. A $CD69^{HI}$, $OCA\text{-}B^{HI}$ population is seen starting at 6 hours of stimulation, which increases with continued stimulation. This pattern recapitulates the data seen in Figure 2.1. The $OCA\text{-}B^{HI}$ $CD69^{LO}$ cells seen after 2-4 hours of stimulation may be a small population of "pre-activated" naïve T cells that were not removed during the enrichment procedures. OCA-B expression seems to precede CD44 surface expression and follow CD62L surface reduction.

Due to the novelty of intracellular OCA-B staining for flow cytometry analysis, it is important for this study, and future studies, to validate the staining and procedures. To do this, I repeated the experiment in Figure 2.2 A, but compared OCA-B expression in T cells isolated from wild-type mice versus $OCA\text{-}B^{-/-}$ mice as a negative control

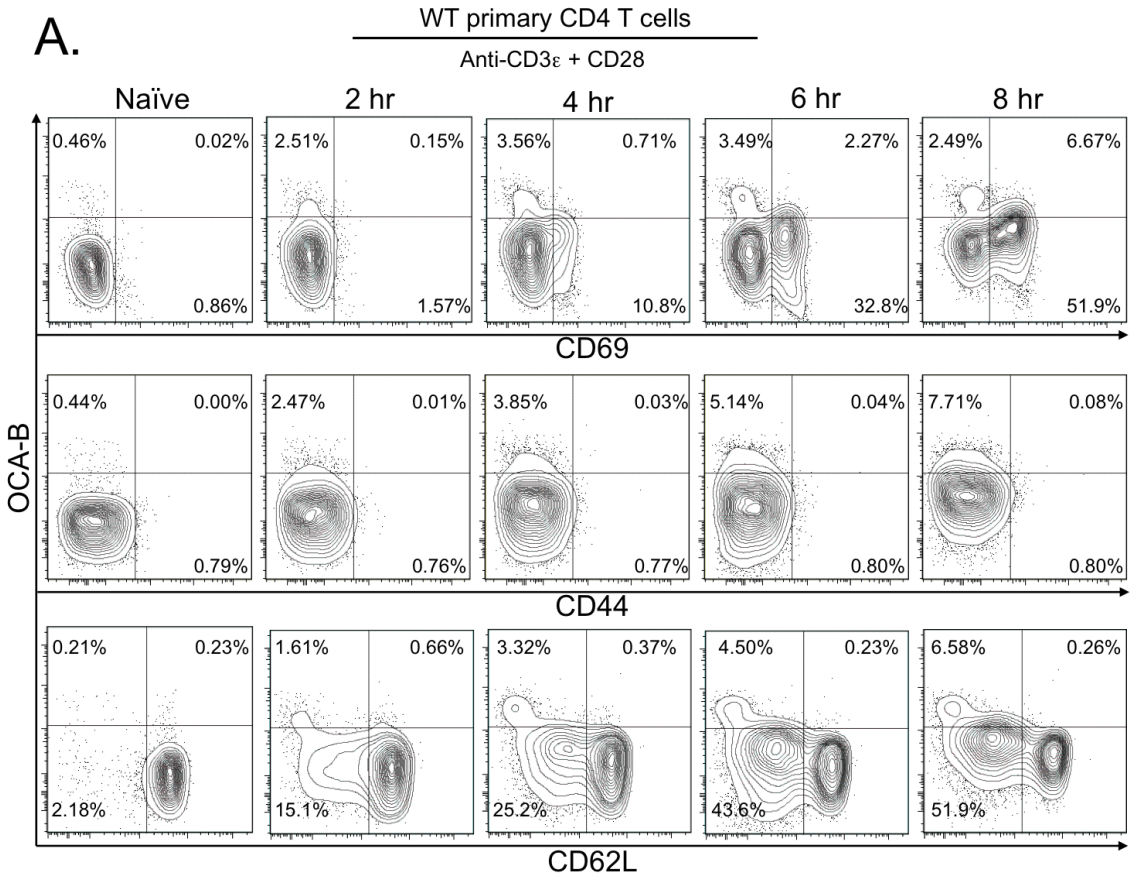

Figure 2.2 Comparing OCA-B induction to CD69, CD62L and CD44 surface markers. A) A duplication of the experiment seen in Figure 2.1, using naïve $CD4^+$ T cells isolated from 3 wild-type C57/BL6 mice with CD3ε/CD28 stimulation times as listed, and an additional surface stain for CD69. Cells displayed are gated on $CD4^+$, $CD8^-$ cells. B) Repeating the experiment comparing naïve $CD4^+$ T cells isolated from 2 wild-type mice and 2 OCA-$B^{-/-}$ mice for validation of antibody staining of intracellular OCA-B. Cells displayed are gated on $CD4^+$, $CD8^-$ cells.

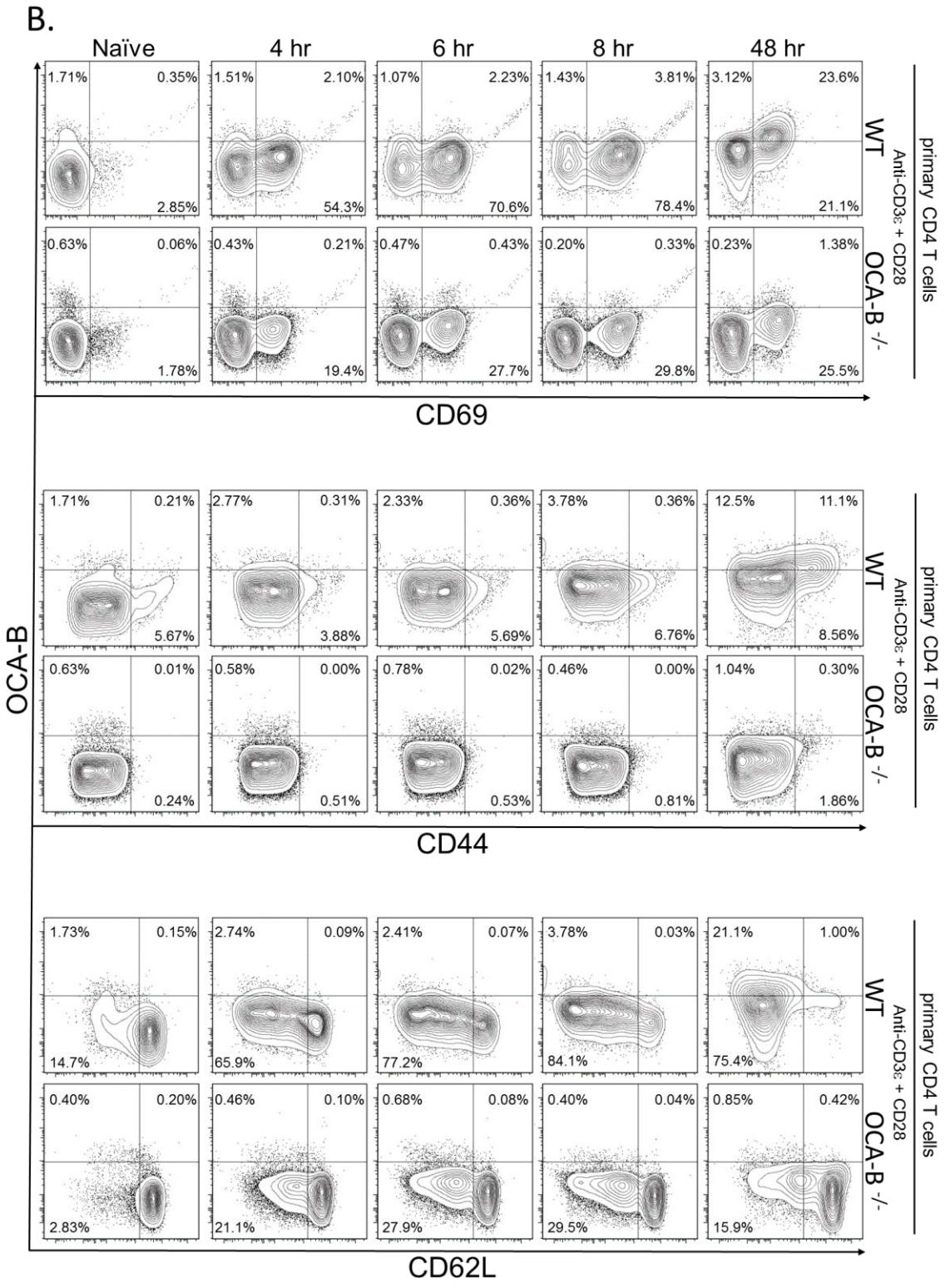

Figure 2.2 Continued

(Figure 2.2B). OCA-B$^{-/-}$ mice show no induction of OCA-B regardless of any amount of stimulation, whereas CD69 and CD62L show expected modulation after stimulation. In addition to CD69 and CD62L modulation, wild-type mice have induction of OCA-B expression seen after 8 hours of stimulation, as seen before. Interestingly, surface CD44 is absent in OCA-B$^{-/-}$ regardless of any amount of stimulation.

Does OCA-B induction require constant stimulation?

It has been shown that development of memory CD4^{+} T cells depends on long, sustained interactions between TCRs and TCR specific antigens (48). With these findings in mind, we hypothesized that sustained stimulation of naïve CD4^{+} T cells is required for OCA-B expression. To test this hypothesis, naïve CD4^{+} T cells were isolated from 3 wild-type mice and plated for various times with CD3ε/CD28 antibodies, washed, and replated without any stimulation. Primary CD4^{+} T cells were stimulated for 6, 8, 10 and 12 hours with overall plate times capped at 12 hours. One aliquot was plated for 12 hours without stimulation. The cells were analyzed via flow cytometry on CD4^{+}, CD8^{-} gated cells (Figure 2.3). OCA-B is found to have a steady increase of expression after 6 hours of stimulation based on the length of stimulation given, in a pattern similar to previous figures.

Oct1 maintains OCA-B expression in activated T cells

Arvind Shakya showed that OCA-B expression is maintained in T cells rested 8 days poststimulation. ((50), Figure 2.1 A). To query if Oct1 is involved in maintaining OCA-B expression, naïve CD4^{+} T cells were isolated from wild-type and Oct1$^{fl/\Delta}$ CD4-

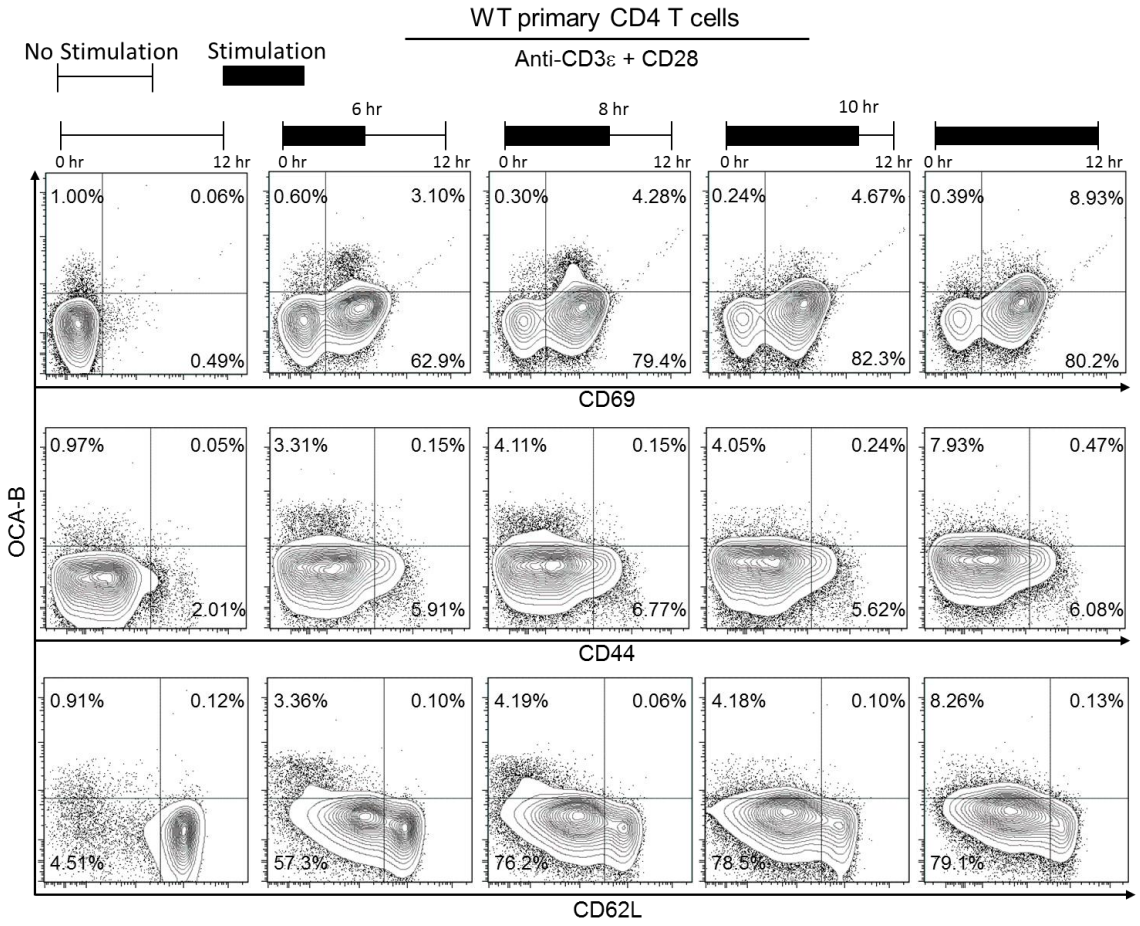

Figure 2.3 Evaluating stimulation requirement for OCA-B induction. Naïve $CD4^+$ T cells isolated from 3 wild-type C57/BL6 mice were plated with or without CD3ε/CD28 stimulation as diagramed above in each column of flow plots. Data shown are on $CD4^+$, $CD8^-$ gated cells.

cre mice, stimulated for 2 days, then rested for 8 days with Il2 in media. OCA-B expression was analyzed by flow cytometry on $CD4^+$, $CD8^-$ gated cells (Figure 2.4). Between the two groups of cells, naïve cells and cells stimulated followed the same induction kinetics for OCA-B, as wells as CD69, CD62L and CD44. After the stimulated cells were rested for 8 days with Il2 in media, OCA-B protein levels were reduced in Oct1 deficient T cells while wild-type T cells maintained OCA-B expression.

Discussion

OCA-B induction after 6-8 hours of stimulation

As shown in my data, and data from previous research efforts, naïve $CD4^+$ T cells do not have endogenous expression of OCA-B (Figure 2.1, Figure 2.2, Figure 2.3, Figure 2.4 (18, 39)). The data presented demonstrate that in vitro CD3ε/CD28 stimulation with antibodies induces detectable OCA-B expression at 6 hours via flow cytometry, which is detectable after 8 hours via western blot. Additionally, OCA-B expression progressively increases with continued stimulation after 6 hours. Compared to activation marker CD69, OCA-B induction is much slower and less robust. After 2 days of stimulation, OCA-B expression has increased beyond levels seen after 12 hours of stimulation, whereas CD69 expression seemingly peaks after 6-8 hours of stimulation and remains at approximately the same level after 2 days of stimulation (Figures 2.2 B, 2.4). This provides some evidence that OCA-B induction is a long, gradual process which may require extended stimulation.

Sauter et al. detects OCA-B mRNA in primary T cells after 1 hour of ionomycin/TPA treatment, with expression grossly peaking after 4 hours (39).

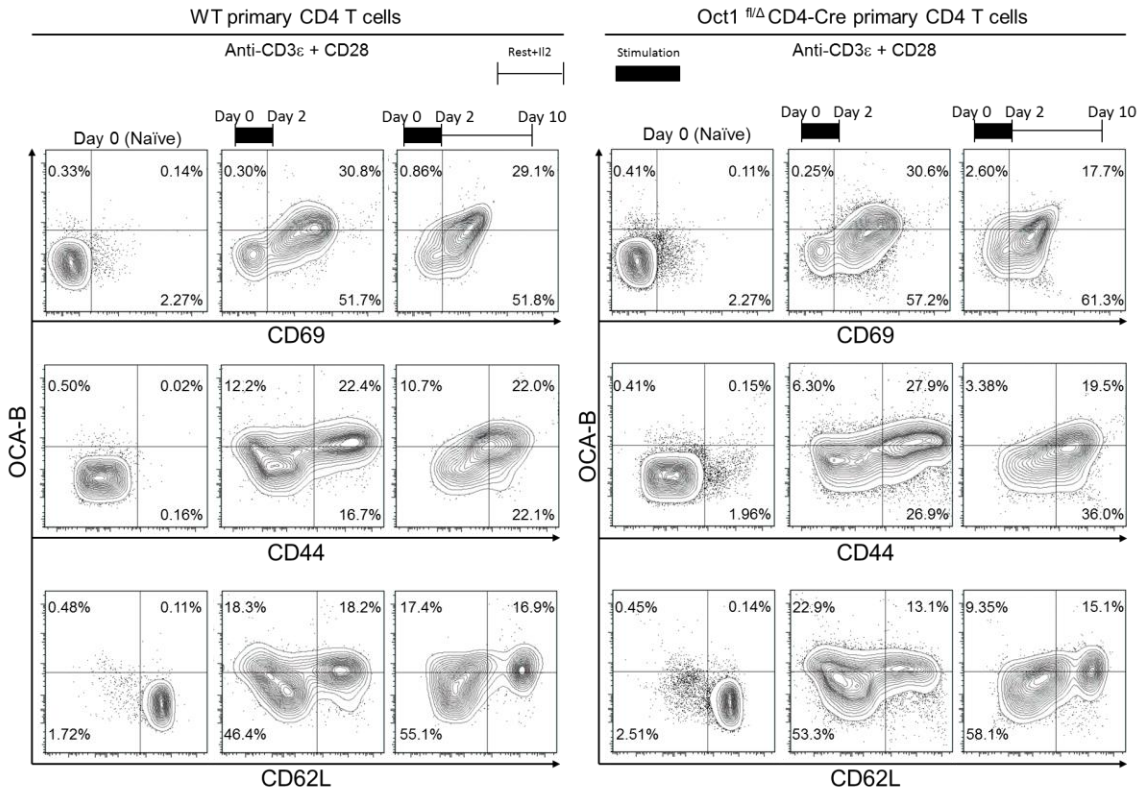

Figure 2.4 Oct1 is involved in OCA-B expression maintenance in activated T cells. Naïve $CD4^+$ T cells were isolated from a wild-type C57/BL6 mouse and an $Oct1^{fl/\Delta}$ CD4-cre mouse. Flow cytometry was performed on sample aliquots taken before stimulation; after 2 days of CD3ε/CD28 stimulation; and after 8 days of rest with Il2 in media, preceded by 2 days of CD3ε/CD28 stimulation, as indicated above in each column of flow plots. $CD4^+$, $CD8^-$ cells gated cells are shown.

Considering OCA-B protein is detected at 6 hours, it seems there is a posttranscription to translation lag of approximately 5 hours. However, ionomycin/TPA treatment bypasses multiple signal transduction steps seen in vivo, activating T cells by means of direct PKC interactions. While it is still an in vitro means to activate T cells, CD3ε/CD28 stimulation using antibodies more closely resembles T cell activation by APCs in vivo and includes more signal transduction steps. With this in mind, and the fact that Sauter et al. did not isolate purely for naïve $CD4^+$ T cells, the lag from RNA to protein is potentially shorter.

OCA-B$^{-/-}$ vs WT naïve T cell stimulation induction profiles

As expected, OCA-$B^{-/-}$ T cells have no OCA-B expression found even after 48 hours of stimulation (Figure 2.2 B). Conversely, OCA-B is significantly stained in wild-type T cells stimulated for 48 hours. It should be noted that there is a slight discrepancy in early OCA-B induction in wild-type $CD4^+$ T cells in Figure 2.2B compared to previous flow plots. The OCA-B detected after 4 hours of stimulation is most likely attributed to the fraction of activated $CD62^{LO}$, $CD44^{HI}$ T cells, as seen in the "naïve" column, which may not have been removed during enrichment.

Interestingly, OCA-B deficient T cells showed no surface expression of CD44 even after 2 days of stimulation. Shakya et al. found that OCA-B deficient $CD4^+$ T cells, isolated form OCA-$B^{-/-}$ mice, have no noticeable decrease in CD44 RNA expression (50). Therefore, OCA-B dependent transcription of *CD44* can be ruled out. CD44 has many isoforms and is posttranslationally modified to direct its function and localization (52). With this in mind, the argument could be made that a separate transacting protein is expressed in an OCA-B dependent manner that posttranscriptionally modifies CD44

appropriately for surface expression. Additionally, since Oct1 deficient T cells have appropriate surface expression of CD44 after 48 hours of stimulation, as seen in Figure 2.4, there is a distinct possibility that CD44 surface localization is dependent on OCA-B with a different transcriptional modifier aside from Oct1. Considering other roles in inflammation, Oct2 could possibly be the alternative transcriptional modulator involved in this mechanism.

The influence of OCA-B on CD44 surface expression is novel and, as such, leads to new lines of inquiry. Primarily, it would be interesting to probe if OCA-B deficient naïve T cells have the same level intracellular CD44 compared to wild-type naïve T cells. If OCA-B deficient naïve T cells exhibit lowered intracellular CD44, then OCA-B may indirectly influence *CD44* translation rather than CD44 posttranslational modifications.

Overall, this finding provides further insight into the role OCA-B plays in memory $CD4^+$ T cell development. Baaten et al. found that engagement of the surface CD44 receptor counteracts Fas mediated apoptosis via P13k/Akt activation, which promotes the long-term survival of memory Th1 T cells (49). If OCA-B truly does effect CD44 surface expression, then it can be deduced that OCA-B indirectly influences the survival of memory T cells.

Induced OCA-B stimulation requirements

By studying TCR/antigen kinetics, Kim et al. found a correlation between TCRs and respective antigens with increased binding length in memory T cells (48). The group concluded that sustained TCR and antigen interactions promote development of memory $CD4^+$ T cells (48). With these findings in mind, we hypothesized that naïve $CD4^+$ T cells

require constant stimulation to induce OCA-B expression. I tested this hypothesis by comparing OCA-B expression in naïve $CD4^+$ T cells stimulated for short periods to cells stimulated for longer windows. Unfortunately, the data gathered have not entirely answered this question and it is still unclear if OCA-B induction requires sustained T cell stimulation. However, given that shorter windowed T cell stimulation closely duplicates the induction patterns of strictly stimulated T cells, the hypothesis cannot be excluded (Figure 2.1 C, Figure 2.2, Figure 2.3). These matching results from the two experimental designs and the noted increase in OCA-B expression between 8 and 48 hours do suggest that constant stimulation may play a role in OCA-B induction.

Kim et al. developed multiple TCR clones with well documented dissociated constants and tetramer binding half-lives. It would be interesting to see if TCR clones identified to generate memory T cell phenotypes have corresponding OCA-B induction. Comparing OCA-B expression in cloned TCRs with longer antigen binding times compared to TCRs with shorter binding times would provide interesting insight into stimulation requirements for OCA-B induction.

OCA-B expression is insulated by Oct1

As previously described, Oct1 modulates genes into active, suppressed or poised expression states. While research has looked into the cooperation of Oct1 and OCA-B as transacting gene regulators, modification of *Pou2af1* expression by Oct1 has not been described. Between Oct1 deficient and wild-type naïve $CD4^+$ T cells, no difference is noted in OCA-B expression after 2 days of stimulation (Figure 2.4). After stimulated cells were plated for 8 days without stimulating antibodies, OCA-B expression in

Oct1deficient $CD4^+$ T cells, specifically $CD44^{HI}$ $CD69^{HI}$ $CD62L^{LO}$ cells, exhibit diminished expression of OCA-B. Wild-type T cells maintain OCA-B expression after rest equal to cells stimulated for 2 days.

In $CD4^+$ T cells selected for memory differentiation, together Oct1 and OCA-B modulate expression and inflammatory gene promoters to prime stimulated T cells for a robust secondary response (50). Here, we see that loss of Oct1 in T cells impairs the ability to maintain OCA-B expression after stimulation. However, 8 days of rest is a relatively short time compared to the longevity of naturally developed memory T cells. As done by Shakya et al., it would be interesting to see how drastically Oct1deficiency diminishes OCA-B expression maintenance in T cells rested for weeks or months post-stimulation. If Oct1 is determined essential for maintenance of long-term OCA-B expression, and given the amount of OCA-B lost 8 days after stimulation, I hypothesize that OCA-B should be absent in T cells rested for 4 to 6 weeks poststimulation. Alternatively, if OCA-B expression is not entirely lost after a long period of rest, it would be interesting to see if OCA-B expression is as robust in Oct1 deficient T cells compared to wild-type T cells in response to secondary stimulation.

OCA-B: memory marker and memory precursor?

Surface expression of CD44 has been previously determined as both a memory T cell marker as well as a receptor required for long-term survival of memory T cells (49). After 12 hours of stimulation, OCA-B is expressed initially in $CD44^{LO}$ T cells (Figure 2.1 C, Figure 2.2). After 48 hours of stimulation, T cells induce CD44 surface expression apparently in an OCA-B dependent manner (Figure 2.2 B, Figure 2.4). It is already

known that Oct1 and OCA-B are essential factors for memory T cell development (50). Providing Oct1 assists in long-term OCA-B expression, and OCA-B feasibly controls CD44 surface expression, it could be inferred that OCA-B expression is indicative of a T cell selected for memory development (Figure 2.4, Figure 2.2). It could be further extrapolated that $CD4^{+}$ "memory precursors" are $CD44^{LO}$, $CD69^{HI}$; and $OCA\text{-}B^{HI}$ T cells that modulate into $CD4^{+}$ memory T cells which are $CD44^{HI}$ $CD69^{HI}$ andwa $OCA\text{-}B^{HI}$.

Conclusions

As shown and discussed, naïve $CD4^{+}$ T cells require 6 hours of CD3ε/CD28 stimulation to begin OCA-B expression. OCA-B induction is slower compared to activation marker CD69, and expression increases gradually with continued stimulation. It has not been fully ascertained if constant stimulation is required for OCA-B expression. However, considering the slow increase of OCA-B expression over time, the hypothesis cannot be ruled out. Maintenance of OCA-B expression is influenced by Oct1, but it is not entirely evident if maintenance is entirely lost in the absence of Oct1.

Interestingly, it seems that OCA-B may influence the surface localization of CD44 in activated T cells. This dependency may indicate the possibility of a $CD44^{LO}$, $CD69^{HI}$, $OCA\text{-}B^{HI}$ pool of "memory precursors" seen in early activation.

Methods

Naïve $CD4^{+}$ T cell isolation

Naïve $CD44^{+}$ T cells were isolated from C57/BL6 mice spleens as described (8). Additional biotin-conjugated CD44 antibody (eBioscience) was used at $0.8ul/10^{8}$ cells.

Cell culture and in vitro stimulation

Isolated T cells were cultured and stimulated with plate bound anti-CD3ε and anti-CD28 antibodies as described (8). Culturing times are as described in the results.

Western blot

Western blot was done using standard procedures. OCA-B blotting was done with rabbit anti-Bob.1 (OCA-B)(c-20) antibody (Bethyl) at a 1:500 dilution in TBST. β-actin blotting was done with mouse anti-β-actin (Abcam) at a 1:1000 dilution in TBST.

Flow cytometry

Flow cytometry surface staining was done following standard procedures using the following stains: Af700-CD4 (1:500); pcpCy5.5-CD8 (1:500); PECy7-CD44 (1:2000); APC-CD62L (1:2000); FITC-CD69 (1:1000). Data were collect using a FacsCantoII and analyzed using FlowJo software (Becton-Dickenson).

For OCA-B T cell intracellular staining, $60x10^4$ to 2×10^4 cells were washed in 200 µl PBS plus 3% fetal bovine serum, and fixed/permeabilized in 100 µL Cytofix/Cytoperm (Becton-Dickenson). Cells were vortexed and incubated for 20 minutes on ice. Subsequently 100 µl of Perm/Wash buffer (Becton-Dickenson) was added, and cells were collected by centrifugation. Cells were resuspended in 100 µL Perm/Wash buffer plus 15 µL anti-OCA-B-PE (Santa Cruz sc-23932), briefly vortexed, and incubated for 40 minutes at 4°C. Following addition of 100 µL of Perm/Wash buffer and collection of cells, the stained cell pellet was resuspended in 200 µL PBS + 3% FBS for analysis.

CHAPTER 3

CONDITIONAL OCA-B KNOCKOUT ALLELE GENERATION

Results

Recombinant mice derived from embryonic stem cells with promotor driven knockout of OCA-B ($Pou2af1^{tm1a(KOMP)Wtsi}$) were purchased from the UC Davis KOMP repository. The OCA-B locus, as generated by UC Davis, includes: an En2 splice acceptor (En2 SA), an internal ribsome entry site (IRES), a beta-galactosidase reporter gene (LacZ), human beta-actin promoter (hBactP), and a neomycin cassette (neo) (Figure 3.1 A (top)). All of these accessory genes are flanked by two FRT recombination sites. As such, the mice were crossed with mice expressing FLP recombinase resulting in a functional OCA-B locus with loxP sites flanking around exons 1, 2 and 3 (Figure 3.1 A (middle)). The resulting "post-FLP" mice will work as the core mice for any future conditional knockout experiments. A truncated knockout of OCA-B results when crossed with a mouse expressing either tissue specific or ubiquitous cre recombinase (Figure 3.1 A (bottom)). Full body knockout ("Null") alleles were generated by crossing post-FLP positive mice with CMV-cre mice, resulting in the truncated OCA-B locus.

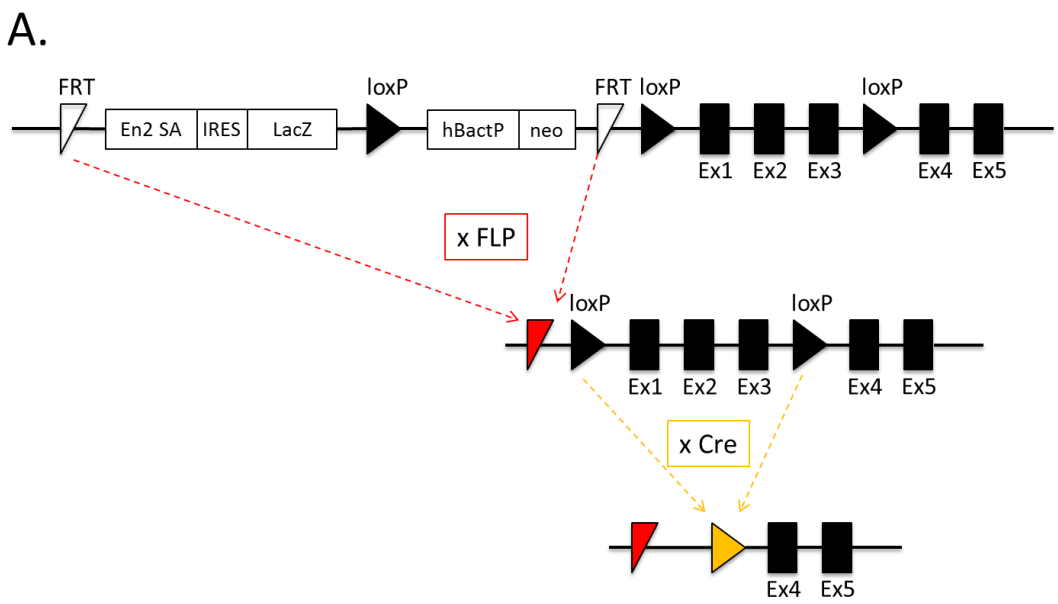

Figure 3.1 Conditional knockout mice genome map and PCR genotyping strategies. A) A representation of the promotor driven conditional knockout OCA-B locus of mice generated from the UC Davis KOMP Repository (top), crossed onto a FLP recombinase mouse (middle), and further crossed to a cre recombinase for a null or tissue specific knockout allele (bottom). B) Diagrams of amplicons resulting from respective PCR primer pairs. C) Representation of amplicons as seen after PCR and ran on 1% agarose gel.

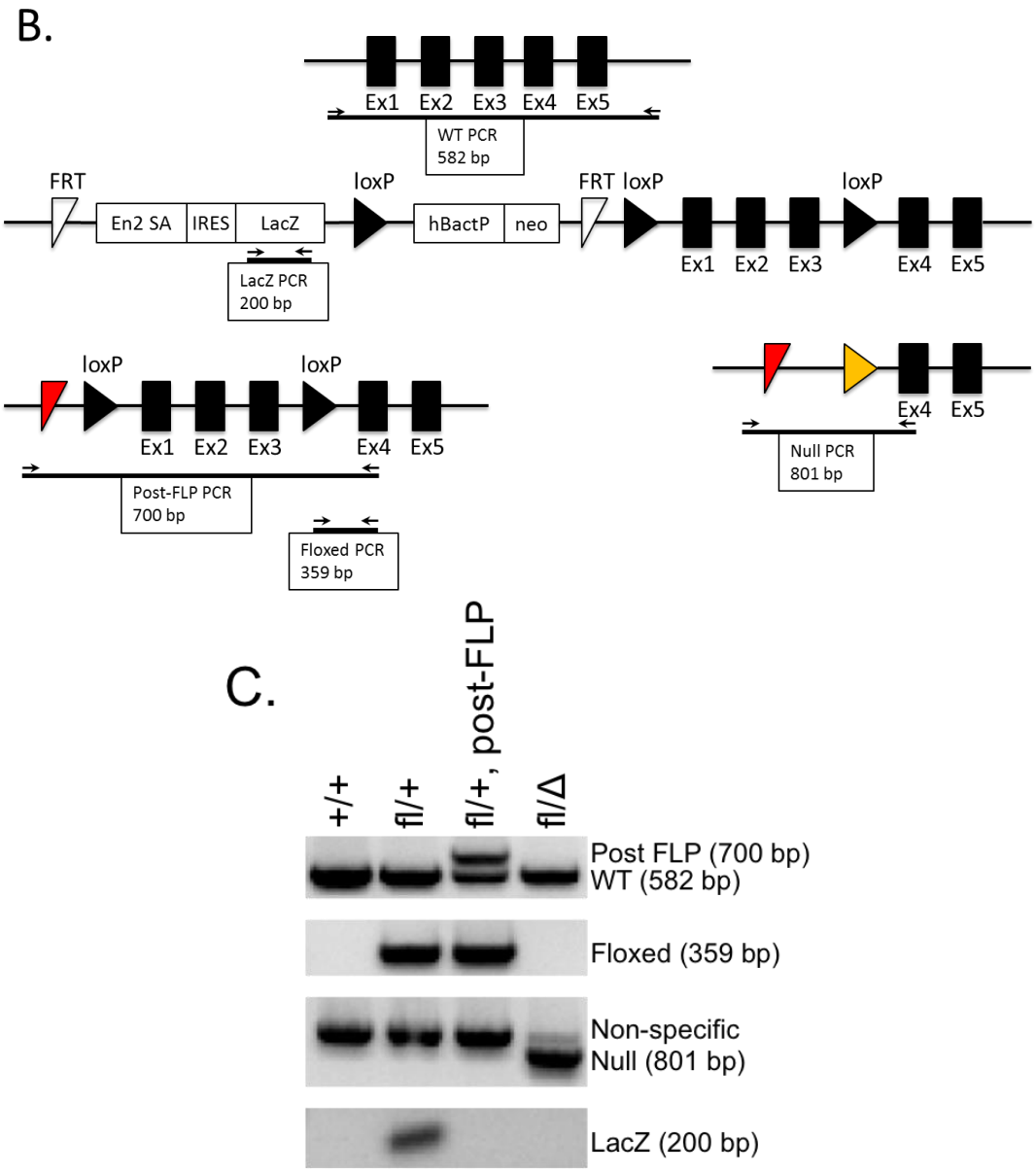

Figure 3.1 Continued

UC Davis KOMP Repository designed primers with theoretical amplicons as represented in Figure 3.1 B. Resulting PCR products of mice currently available are as represented in Figure 3.1 C. It is important to note that the Null primer set gives an unidentified, nonspecific band at approximately 1000 base pairs. According to UC Davis representatives, there is nothing at this locus which could result in such a band. As long as care is taken to run the gel accordingly, this nonspecific band is easily distinguishable from the null allele.

Overall, the generated heterozygous progeny display no gross abnormalities. Generation of OCA-$B^{fl/fl}$, OCA-$B^{fl/\Delta}$, and OCA-$B^{\Delta/\Delta}$ animals is underway, but considering the viability of OCA-$B^{-/-}$ mice as seen in other experiments, any homozygous allele is expected to have a viable phenotype. Provided that it demonstrates OCA-B sufficiency (e.g., protein expression, germinal center formation), the floxed OCA-B allele isolated in this project will be an invaluable tool for further researching the role OCA-B has, not only in memory T cell differentiation, but also in OCA-B related hematopoietic disease states.

Methods

OCA-B specific PCR recipe and parameters

DNA was isolated from mice tail tips using standard HotSHOT tail DNA preparation procedures. Genotype protocols and primer designs were as provided from UC Davis Komp repository.

A 1.5 µL sample of DNA was combined with the following: 9.08 µL autoclaved dH_2O; 6.5 µL Betaine (Sigma); 0.33 µL DMSO; 5.0 µL 5x Phire HotStart reaction buffer

(Life Technologies); 0.4 µL 10 mM dNTPs; 1.0 µL forward primer; 1.0 µL reverse primer; 0.20 µL Phire HotStart Taq Pol II (Life Technologies).

The PCR cycling parameters for each genotype are as follows: 94°C for 5 minutes; 94°C for 15 seconds to 65°C for 30 seconds to 72°C for 40 seconds repeated 10 times with a 1°C decrease per cycle; 94°C for 15 seconds to 55°C for 30 seconds to72°C for 40 seconds repeated 30 times; 72°C for 5 minutes; 4°C until removed for analysis.

OCA-B primer design

Primers for wild-type OCA-B and post-FLP recombination are as follows: CSD-OCAB-F (aka. CSD-Pou2af1-F) 5'- TACAGAGAGACTAGACACGGTCTGC-3' and CSD-OCAB-ttR (aka. CSD-Pou2af1-ttR) 5'-AGAAGGCCTCGTTACACTCCTATGC-3'.

Primers for floxed OCA-B are as follows: CSD-Lox-F 5'-GAGATGGCGCAACGCAATTAATG-3' and CSD-OCAB-R (aka. CSD-Pou2af1-R) 5'-GATGAGGACTCTGGGTTCAGAGAGG-3'.

Primers for null OCA-B are as follows: CSD-OCAB-F (aka. CSD-Pou2af1-F), 5'-TACAGAGAGACTAGACACGGTCTGC-3' and CSD-OCAB-R (aka. CSD-Pou2af1-R) 5'- GATGAGGACTCTGGGTTCAGAGAGG-3'.

LacZ PCR recipe and parameters

A 1.5 µL sample of DNA was combined with the following: 16.9 µL autoclaved dH_2O; 5.0 µL 5x Phire HotStart reaction buffer (Life Technologies); 0.4 of 10 mM dNTPs; 0.5 µL forward primer; 0.5 µL reverse primer; 0.20 µL Phire HotStart Taq Pol II

(Life Technologies).

The PCR cycling parameters for LacZ PCR are as follows: 94°C for 5 minutes; 94°C for 20 seconds to 59°C for 20 seconds to 72°C for 40 seconds repeated 29 times; 72°C for 10 minutes; 4°C until removed for analysis.

LacZ primers were provided by Arvind Shakya of the University of Utah Tantin Lab.

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